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Determination of acidity constants at 37 ºC through the internal standard capillary electrophoresis (IS-CE) method: internal standards and application to polyprotic drugs

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This work provides the pK_a at the biorelevant temperature of 37 ºC for a set of compounds proposed as internal standards for the internal standard capillary electrophoresis (IS-CE) method. This is a high throughput method that allows the determination of the acidity constants of compounds in a short time, avoiding the exact measurement of the pH of the buffers used. pH electrode calibration at 37 ºC can be avoided too.

In order to anchor the pK_a values obtained through the IS-CE method in the pH scale, the acidity constant at 37 ºC of some of the standards has been determined also by the reference potentiometric method. In general, a decrease in the pK_a value is observed when changing from 25 to 37 ºC, and the magnitude of the change depends on the nature of the compounds.

Once the pK_a values at 37 ºC of the internal standards have been established, the method is applied to the determination of the acidity constants of seven polyprotic (5 diprotic and 2 triprotic) drugs. The obtained mobility-pH profiles show well defined curves, and the fits provide precise pK_a values. Due to the lack of reference data at 37 ºC only the pK_as of labetalol can be compared to values from literature, and a very good agreement is observed.

Introduction

In the past decades, pharmaceutical industry has undergone considerable changes due to application of many cutting-edge technologies such as combinatorial chemistry, high throughput screening, and robotics, and all these new technologies have lead to the possibility to synthetize millions of compounds for a high number of different health conditions and diseases. The drug discovery process is very complex, expensive, and time-consuming; in average, it takes from 10 to 15 years from the discovery of a new compound until the new drug is released to the market. The development of better screening methods for the evaluation of physicochemical properties of candidate compounds at the early stage of the drug discovery process can reduce time, decrease expenses, and diminish the number of failures in the late stage of drug development. Therefore, there is a great need for high-throughput screening methods for a rapid determination of physicochemical properties of candidate drug compounds.[1-3]

One of the properties that affects the pharmaceutical potential of a compound, among others, is the acidity constant. The acidity constant determines the degree of ionization of the compound, which has impact on its solubility, dissolution rate, absorption across biological membranes (lipophilicity and permeability), distribution to the site of action, renal elimination, interaction with efflux systems, metabolism, protein binding, and receptor interactions.[2,4-5] Nonetheless, most of the acidity constants are determined at 25 °C or at room temperature, which usually can range from 15 to 30 °C, instead of at 37 ºC, the common human biorelevant temperature. Accurate determinations of the pK_a values at 37 °C is important for all drugs introduced orally, intramuscularly or any other way in which candidate compound should be absorbed, distributed, metabolized or excreted at the normal body temperature. Acidity constant, pK_a in its logarithmic form, depends on temperature (T), and many studies demonstrate that change of pK_a value due to temperature depends on the nature of the functional groups of the compounds. Whereas simple carboxylic acids have nearly the same pK_a value at 25 and 37 °C, the pK_a values of some bases or phenols show an important temperature dependence.[5-10] For example, quetiapine, a basic anti-psychotic drug, with pK_{a1} = 2.27 and pK_{a2} = 7.30 at 25 ºC[11] changes its pK_a values to 3.56 and 6.38 at 37 ºC[5], respectively. Although the variation of the second pK_a is not as big as for the first pK_a value, its proximity to the physiological pH of 7.4 makes accurate determination of pK_a value even more important. Thus, the ionization degree of quetiapine at physiological conditions of pH will be lower at 37ºC than at 25 ºC, fact that can lead to wrong biodisponibility conclusions if the considered pK_a values are the ones at 25ºC.

This work is focused on the use of the internal standard capillary electrophoresis method (IS-CE) for the determination

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of pK_a values at 37 ºC. CE has several advantages compared to other methods for the determination of acidity constants. It is a highly automated technique that requires small amounts of compounds and where high purity is not required due to the separation performed inside the capillary.[12-19] The IS-CE method is based on the use of an internal standard (i.e. a compound with known pK_a) to measure the pH inside the capillary. This is obtained from the pK_a value of the IS and the ratio of mobilities of the pure ionic form of the IS, and the mobility at a pH where it is partially ionized. Once the pH is known, the ratio of mobilities of the test compound (fully and partially ionized) is used to determine its pK_a.[20,21] Compared to the classical CE method for determination of acidity constants, the IS-CE is much faster, and it does not require a previous exact measurement of the pH value of the buffer, since the pH is measured in situ in the capillary under the exact conditions of analysis. This is especially relevant in measurements at a given temperature, since the calibration of the electrodic system used to measure the pH of the buffers at the working temperature can be avoided. Moreover, previous works have demonstrated that if the IS and the analyte behave in a similar way, possible alterations in the experimental conditions that may affect the mobilities of the test compound (as for example Joule effect) can be compensated by the IS.[6,22] On the contrary, in the classical CE method any alteration of the mobility of the analyte leads to a direct error in the pK_a determination.[14]

The IS-CE method has been well established for the determination of pK_a values of all types of acid-base compounds at 25 ºC. Nevertheless, there is a lack of pK_a values for internal standards at 37 ºC. The main purpose of this work is, therefore, to establish the pK_a values at 37 ºC for a reference set of internal standards in order to make this high-throughput method applicable to the characterization of drugs at the human biorelevant temperature.

Theory

For an ionizable acid–base compound, the electrophoretic mobility depends on the degree (or degrees) of ionization of the compound. Thus, the general acid–base equilibria for a monoprotic species, HX^z, can be expressed as:

$$HX^z \rightleftarrows X^{z-1} + H^+ \qquad \mathbf{pK'_a = pH - \log \frac{[X^{z-1}]}{[HX^z]}} \qquad (1)$$

where z is the charge number of the protonated species and pK_a' the logarithmic form of the mixed acidity constant (at a given ionic strength, as well as temperature and solvent).[23,24]

The μ_{ep}, also called the effective mobility (μ_{eff}), of a monoprotic compound can be expressed as a function of the pK_a' of the species and the pH of the background electrolyte through the following general equation[13]:

$$\mathbf{\mu_{eff} = \frac{\mu_{HX^z} + (\mu_{X^{z-1}})10^{pH-pK'_a}}{1 + 10^{pH-pK'_a}}} \qquad (2)$$

where $\mathbf{\mu_{HX^z}}$ and $\mathbf{\mu_{X^{z-1}}}$ are the actual ionic electrophoretic mobilities of the subscripted species.

In the IS-CE method, the use of an IS allows direct calculation of the pH of the buffered solution inside the capillary. This value is then used to calculate pK_a' of the compound being studied.

For example, for a neutral acid used as the IS, where z is 0, the pH value can be given, by rearranging Eq. 2, by:

$$\mathbf{pH = pK'_{a(IS)} - \log \frac{\mu_{IS^-} - \mu_{eff(IS)}}{\mu_{eff(IS)}}} \qquad (3)$$

where $\mathbf{\mu_{eff(IS)}}$ is the effective mobility and $\mathbf{\mu_{IS^-}}$ the actual ionic mobility (mobility of the fully charged negative species) of the IS. The term corresponding to the uncharged species, $\mathbf{\mu_{HX}}$, has zero mobility and is removed from Eq 2. Since $\mathbf{pK'_{a(IS)}}$ of the IS is well known, only two runs at two different pH values are needed to calculate the pH: the first where the pH is in the range $pK_a \pm 1$, in order to calculate the effective mobility; and a second in which it is completely ionized to calculate the actual ionic mobility. Therefore, although a buffer could experiment pK_a shifts due to temperature, the provided pH inside the capillary can be known by the relation of mobilities and pK_a of the IS.

Once the pH inside the capillary is known, the acidity constant of the test compound (TC) can be calculated by rearranging Eq. 2 again:

$$\mathbf{pK'_{a(TC)} = pH - \log \frac{\mu_{HX^z} - \mu_{eff(TC)}}{\mu_{eff(TC)} - \mu_{X^{z-1}}}} \qquad (4)$$

where $\mathbf{\mu_{HX^z}}$ and $\mathbf{\mu_{X^{z-1}}}$ refer to the actual ionic mobilities of the fully charged z and z-1 species of the TC.

In these equations, $\mathbf{pK'_{a(TC)}}$ is related to the thermodynamic pK_a by the activity coefficients, usually calculated by means of the Debye-Hückel equation (Eq. 5)[21]:

$$\mathbf{-log\,\gamma = \frac{Az^2\sqrt{I}}{1+Ba\sqrt{I}}} \qquad (5)$$

I is the ionic strength of the buffer solutions (0.05 M in this work), A and B depend on the solvent relative permittivity and temperature (their values are 0.523 and 0.331, respectively, in water at 37 ºC), z is the charge number of the ion, and a is the hydrated radius of the ion. The value of a depends on the hydrated ion, although a value of 4.5 Å (value for hydrogen ion) is commonly taken for most ions. This equation is valid for ionic strength values lower than 0.2 M. Activity coefficients of neutral species (z = 0) are assumed to be unity.

In case of polyprotic compounds, equation 2 becomes equation 6:

$$\mu_{eff} = \frac{\mu_{H_nX^z} + \sum_{i=1}^{n} 10^{ipH - \sum_{j=1}^{i} pK'_{aj}} \mu_{H_{n-i}X^{z-i}}}{1 + \sum_{i=1}^{n} 10^{ipH - \sum_{j=1}^{i} pK'_{aj}}} \quad (6)$$

This is a general expression that relates effective mobility and pH for any type of acid-base compound.

Electrophoretic mobility values are calculated through the migration times of the analyte (t_m) and the electroosmotic flow marker (t_0) according to equation 7:

$$\mu_{ep} = \frac{L_T L_D}{V}\left(\frac{1}{t_m} - \frac{1}{t_0}\right) \quad (7)$$

where L_T and L_D are the total and effective capillary length, respectively, and V is the applied voltage. Mobilities obtained in this way can be directly used only in absence of Joule heating effect, which causes an increase of temperature inside the capillary above the temperature of the capillary coolant. Otherwise, temperature corrections of measured mobilities must be applied. [25,26]

Experimental

Apparatus and conditions.

CE determinations were performed in a P/ACE MDQ Beckman instrument (Palo Alto, CA, USA) equipped with a diode-array spectrophotometric detector. A fused-silica capillary of 50 µm i.d. and 375 µm o.d. from Polymicro Technologies (Phoenix, AZ, USA) was used. Capillary had a total length of 35.2 cm, and an effective length of 25.0 cm. Samples were injected at a hydrodynamic pressure of 2068 Pa (0.3 p.s.i.) during 2.0 s. Separation was performed applying a pressure of 3447 Pa (0.5 p.s.i.) and a voltage of 20 kV. The absence of Joule effect at these separation conditions has been tested for each of the used buffers, by checking the linearity between the applied voltage and the generated current according Ohm's law. Detection was carried out at 214, 254 and 280 nm with full spectra captured from 190 until 300nm. Capillary together with sample tray were thermostated at 37 °C (± 0.1 ºC). Under these conditions electrophoretic runs duration was under 5 minutes in most of the buffers.
Potentiometric pK_a determinations were performed in an 888 Titrando potentiometer from Metrohm (Herisau, Switzerland), equipped with a combined pH electrode and a burette also from Metrohm, a tempering beaker, and a temperature-controlled water bath (J. P. Selecta, Abrera, Spain).

Reagents and materials.

Sodium hydroxide (0.5M TitrisolTM) and hydrochloric acid (1M TitrisolTM), dimethyl sulfoxide (>99.8%), and sodium dihydrogen phosphate monohydrate (>99%) were from Merck (Darmstadt, Germany). Methanol (HPLC grade) was from Thermo Fischer Scientific (Waltham, MA, USA). Sodium acetate anhydrous (>99.6%) was from Baker (Center Valley, PA, USA). Potassium hydrogen phthalate (>99.9%), BisTris (2,2-Bis(hydroxymethyl)-2,2',2''-nitrilotriethanol) (>99%), Tris (Tris(hydroxymethyl) aminomethane) (>99.9%), CHES (2(cyclohexylamino)ethanesulfonic acid) (>99%), and CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) (>98%) were from Sigma-Aldrich (St Louis, MO, USA). Water was purified by a Milli-Q plus system from Millipore (Bedford, MA, USA), with a resistivity of 18.2 MΩ cm.

All internal standards and drugs used for the determinations were obtained from Sigma-Aldrich, Scharlab (Sentmenat, Spain), Merck, Panreac (Castellar del Vallès, Spain), Carlo Erba (Milano, Italy), and J. T. Baker with purity above 98%.

Procedures.

CE capillary conditioning. Capillary conditioning methodology was reported previously.[21] Briefly, before the first use it was conditioned with 1M NaOH, water and the running buffer; when the buffer was changed it was rinsed with water and the new buffer; and between runs it was rinsed for 3 minutes with the running buffer. At the end of the working session the capillary was flushed with water.

Preparation of buffers and solutes for CE analysis. Buffer solutions covering practically all the useful pH range (from 2.0 to 12.0 separated at intervals of 0.5 pH units) were prepared at the approximate pH and 50 mM ionic strength as described elsewhere.[21]
Stock solutions of test compounds and ISs were prepared at a concentration of 1 mg mL^{-1} and 2% v/v of DMSO was added as electroosmotic flow (EOF) marker. They were diluted in water or in a methanol/water mixture (when they were not soluble in water itself). Afterwards, a 1/10 dilution of the stock solution in water was prepared for injection (100 mg L^{-1}, 0.2% v/v DMSO). Internal standards and test compounds were stored at 4ºC until used. All the samples and buffers were filtered through a nylon mesh 0.45 µm porous size (Whatman, Maidstone, UK).

Potentiometric determination of pK_a values. 30 mL of an approximately 0.005 M aqueous solution of the IS were placed in the thermostated beaker for the titration. Once the solution had reached 37 ºC, the titration was performed using 0.1 M sodium hydroxide or 0.1 M hydrochloric acid, depending on the nature of the IS from pH 2 to pH 12, or vice versa. All solutions (titrands and titrants) were prepared with boiled water. 0.1M sodium hydroxide solution was previously standardized with potassium hydrogen phthalate. 0.1 M hydrochloric acid solution was standardized using Tris as primary standard. The potentiometric system was calibrated with standard reference solutions at pH 2, 4, 7, and 9 at 37 ºC. pK_a was calculated through the titration data, taking into account the mass and charge balances of the species in equilibrium. Activity corrections at each titration point were

done through the activity coefficients, which were calculated through the Debye-Hückel equation.

Data analysis.

Table Curve 2D from Systat Software Inc. (San Jose, CA, USA) was used to fit the mobility – pH curves of polyprotic drugs. Other data calculations were performed through Excel 2010 from Microsoft (Redmond, WA, USA).

Results and discussion

Determination of the pK_a at 37 ºC for the set of internal standards.

In order to establish IS-CE as a high-throughput method for determination of pK_a values at 37 °C it is necessary to precisely determine pK_a values of internal standards at this temperature. For this purpose, the previously established set of 23 monoprotic acids and 22 monoprotic bases[22,27] has been used. These compounds have different chemical properties and cover the most useful pH range in CE. Table 1 lists the set

Table 1 pK_a values for the set of internal standards. Standard deviations are shown in parentheses.

Internal Standards	pK_a 25 ºC[22,27]	N	Reference pK_a 37 ºC	IS-CE pK_a 37 ºC
2-Chlorobenzoic acid (A)	2.84 (0.02)	6		2.79 (0.01)
2,6-Dibromo-4-nitrophenol (A)	3.31 (0.03)	7		3.24 (0.03)
4-Nitrobenzoic acid (A)	3.37 (0.01)	7	3.44[28]	3.27 (0.04)
2,6-Dinitrophenol (A)	3.69 (0.01)	7		3.57 (0.04)
3-Bromobenzoic acid (A)	3.79 (0.02)	6		3.71 (0.05)
2,4-Dinitrophenol (A)	4.12 (0.02)	8		3.89 (0.07)
Benzoic acid (A)	4.22 (0.03)	18	4.24[5]	4.07 (0.07)
Ibuprofen (A)	4.49 (0.02)	24	4.51[29]	4.37 (0.06)
Aniline (B)	4.63 (0.02)	14	4.41 (0.03)	4.53 (0.05)
4-*tert*-Butylaniline (B)	4.93 (0.01)	22		4.56 (0.07)
Quinoline (B)	4.93 (0.01)	14	4.73 (0.02)	4.86 (0.03)
Warfarin (A)	5.17 (0.04)	11		4.79 (0.06)
N,*N*-Dimethyl-*N*-phenylamine (B)	5.17 (0.02)	6		5.32 (0.12)
Pyridine (B)	5.28 (0.01)	7	5.09[30]	5.14 (0.04)
2,5-Dinitrophenol (A)	5.30 (0.05)	11		5.05 (0.06)
Sulfacetamide (B)	5.42 (0.05)	14		5.25 (0.05)
Acridine (B)	5.55 (0.06)	9	5.01[31]	5.37 (0.07)
4-*tert*-Butylpyridine (B)	6.03 (0.03)	13		5.92 (0.06)
2,4,6-Tribromophenol (A)	6.04 (0.08)	13		5.88 (0.05)
Papaverine (B)	6.41 (0.07)	8	6.22[32]	6.32 (0.04)
2,4-Lutidine (B)	6.81 (0.05)	19		6.66 (0.06)
Trazodone (B)	6.84 (0.05)	11		6.69 (0.04)
Pilocarpine (B)	7.08 (0.02)	12	6.68 (0.01)	6.85 (0.03)
4-Nitrophenol (A)	7.09 (0.05)	16	7.08 (0.01)	6.82 (0.06)
Vanillin (A)	7.36 (0.06)	21		7.16 (0.05)
2,4,6-Trimethylpyridine (B)	7.51 (0.03)	10	7.16 (0.02)	7.22 (0.07)
Phenobarbital (A)	7.53 (0.04)	22		7.17 (0.05)
4-Hydroxybenzaldehyde (A)	7.61 (0.04)	10		7.35 (0.05)
Lidocaine (B)	7.93 (0.01)	14	7.56 (0.01)	7.78 (0.05)
3,5-Dichlorophenol (A)	8.18 (0.04)	11	8.11 (0.01)	7.92 (0.05)
Bupivacaine (B)	8.19 (0.03)	10	7.97[33]	7.96 (0.07)
Methylparaben (A)	8.35 (0.03)	16	8.21 (0.01)	8.14 (0.06)
2-Chlorophenol (A)	8.50 (0.04)	15		8.22 (0.06)

1-Phenylpiperazine (B)	8.75 (0.02)	9	8.33 (0.02)	8.41 (0.07)
N,*N*-dimethyl-*N*-benzylamine (B)	8.95 (0.04)	11	8.64 (0.04)	8.76 (0.05)
3-Chlorophenol (A)	9.04 (0.01)	16		8.73 (0.06)
Diphenhydramine (B)	9.08 (0.02)	15	8.85[5]	8.83 (0.06)
4-Bromophenol (A)	9.28 (0.01)	27		9.01 (0.05)
Imipramine (B)	9.37 (0.02)	17	9.23[34]	9.25 (0.05)
Propranolol (B)	9.47 (0.00)	23	9.17[32]	9.24 (0.08)
1-Aminoethylbenzene (B)	9.52 (0.01)	11		9.34 (0.10)
Paracetamol (A)	9.58 (0.02)	11	9.65[35]	9.31 (0.03)
Ephedrine (B)	9.72 (0.02)	12	9.12 (0.01)	9.27 (0.04)
Phenol (A)	9.89 (0.01)	13	9.83 (0.01)	9.54 (0.06)
Nortriptyline (B)	10.08 (0.01)	5		9.79 (0.04)

of internal standards, together with their thermodynamic pK_a values at 25 ºC.

A similar approach as the one used at 25 ºC has been applied to determine the new pK_a values.[22,27] Compounds with pK_a values differing less than one unit have been paired, and the roles of test compound and internal standard assigned. For this initial experimental design, pK_a values at 25 ºC have been used when reference data at 37 ºC (Reference pK_a 37 ºC in Table 1) are not available. Then, each pair has been injected twice in the system at 37 ºC. Firstly, using a buffer at a pH value where both compounds are partially ionized to obtain the effective mobilities. Secondly, in a buffer at a pH value where they are totally ionized, to obtain the actual ionic mobilities. In case of pairs of different acid-base nature, actual ionic mobilities have been obtained at two pH values (2.0 for acids and 12.0 for bases). In several instances the same pair has been injected at different pH values for the effective mobility determination, which rises the number of independent experiments (N) for each compound.

Once mobilities are calculated, Eq. 4 has been applied to each pair. To calculate the pK_a of the test compound, obtained mobility data and the pK_a at 25 ºC (or at 37 ºC when possible) of the compound used as internal standard have been used. In this way, N new pK_a values have been obtained for a given compound of the set. The average of these N pK_as provides a new value to start the iterative process, in which the same electrophoretic data is used, but the pK_as of the ISs are changed by the averaged values. The process has been repeated four times, until the differences between consecutive averaged pK_as has been 0.02 pK_a units or less. Once the iteration process has finished, boxplots have been used to identify possible outliers within the N pK_a values obtained for a same compound. Tukey's criterium has been used to remove the outliers.

Next step is to anchor the pK_a scale by comparison of the obtained pK_a values to the known reference values. As accurate literature pK_a data at 37 ºC was scarce for the set of compounds used in this work, potentiometric determinations at 37 ºC have been performed for some of the compounds in order to widen the available reference data. A linear trend is observed when the raw CE data is compared to the potentiometric data, according to Eq. 8:

$$pK_{a,IS\text{-}CE,raw} = 0.165(0.140) + 0.986(0.019)\ pK_{a,Ref} \quad (8)$$

n= 21; SD = 0.170; F = 2925

here $pK_{a,IS\text{-}CE\ raw}$ is the pK_a obtained through the electrophoretic method just after the refinement process, $pK_{a,Ref}$ is the pK_a determined potentiometrically or obtained from the literature, n is the number of compounds used for the correlation, SD is the standard deviation of the correlation, and F is the Fisher F parameter.

This correlation indicates that both set of values are equivalent, since by Student's t-test at 95% confidence level, the slope is not significantly different from 1 and the intercept

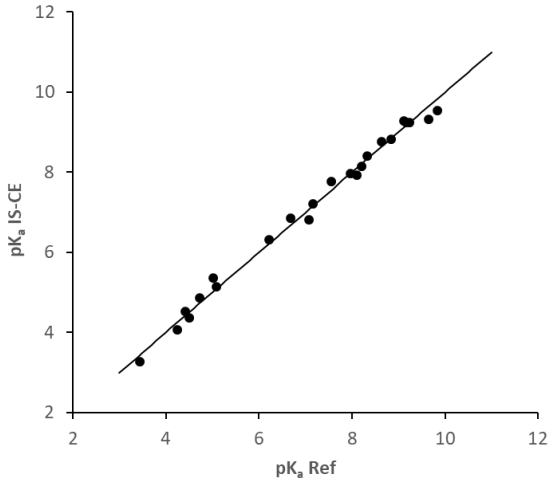

Fig. 1 pK_a values at 37 ºC obtained through the IS-CE method vs. the reference potentiometric ones. Solid line has an ordinate of 0 and a slope of 1.

from 0. Thus, the pK_a values obtained follow a variation of acidity close to the reference one (slope one) and show a similar accuracy (intercept zero). However, for a best anchoring of the CE data to the potentiometric pK_a scale, the differences between IS-CE and reference values have been calculated for each compound ($pK_{a,Ref} - pK_{a,IS\text{-}CE\ raw}$), obtaining an average difference of -0.063 units (which would correspond to the intercept of the line of slope one). Then, the electrophoretic data has been corrected by this value to obtain the final values presented in Table 1. The correlation between the IS-CE measured and reference potentiometric values is presented in Figure 1. This set of final pK_a values can be used for determination of the pK_a at 37 ºC of new compounds through the IS-CE method.

Determination of pK_a at 37 ºC of polyprotic drugs through the IS-CE method.

The method has been applied to the determination of acidity constants of seven polyprotic drugs. The selected drugs are of different nature and show a wide range of acid-base functionalities. Their structures are plotted in Figure 2. Although these molecules show multiple acid-base groups, only those pK_a values ranging between 2 and 11 can be determined through electrophoretic methods.

In order to apply the IS-CE method, a first estimation of the pK_a values of the drugs is needed. This can be easily done through estimation software, such as ACD/Percepta.[36] This information allows the selection of the most appropriate standards (those Then, mixtures of the drug with each of the standards have been done, and electrophoretic runs performed. Effective mobilities have been measured at least in three different buffers (normally differing in 0.5 pH units) for each drug-internal standard pair. Additionally, actual ionic mobilities of the internal standards have also been determined to calculate the real pH value inside the capillary. When possible, actual ionic mobilities of the pure species of drug compounds have been measured too. To decide whether actual ionic mobilities of the drugs could be experimentally determined, the estimated pK_a values at 25 ºC were used. In case that two consecutive pK_as were separated enough (for instance the case of procainamide) or the pK_a of the most charged species was not at very extreme pH values (like cephalexin) the experimental determination of the actual ionic mobilities was done. Otherwise, they have been calculated in the fitting process. Once the real pH inside the capillary is known, the mobility vs. pH curve for the drugs can be plotted. Equation 6 is then fitted to experimental points in order to obtain the pK_a values. Figure 2 shows the different curves, and Table 2 the results obtained for the selected drugs together with the statistics of the fits (R^2 is the determination coefficient, SD the standard error of the fit, and F the Fisher F parameter). pK_a values have been corrected using the Debye-Hückel equation (eq. 5) for 50 mM ionic strength, so that values given correspond to thermodynamic quantities.

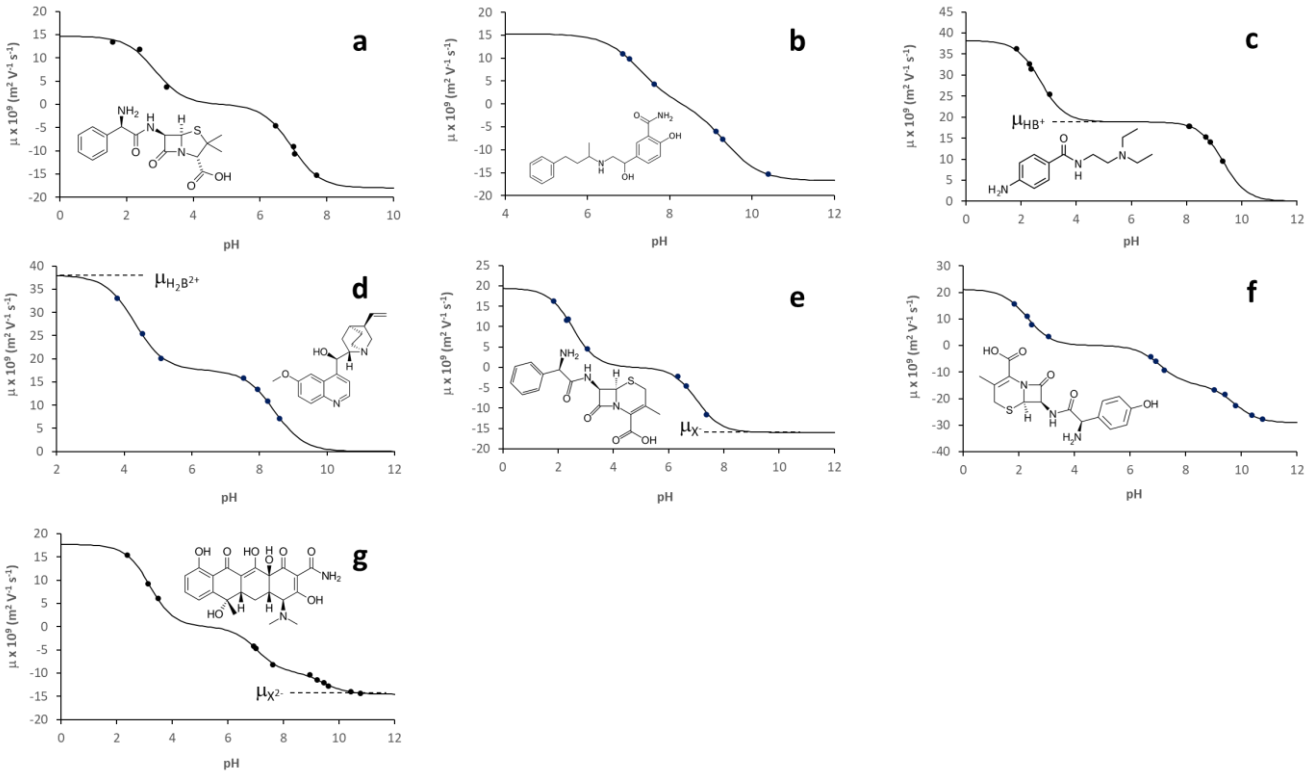

Fig. 2 Mobility vs. pH profiles of the polyprotic drugs. Dots indicate the experimental points and the curves the fit of Eq. 6 to experimental data. Dashed lines indicate the experimentally measured mobilities. (a) ampicillin; (b) labetalol; (c) procainamide; (d) quinine; (e) cephalexin; (f) cefadroxil; (g) tetracycline.

Table 2 pK_a values at 37 ºC for the polyprotic drugs determined through the IS-CE method. Standard deviation is shown in parentheses.

Drug	Acid-base group	pK_a	μ_{ion} x 10^9 [a]	z[b]	Statistics of the fit		
					R^2	SD	F
Ampicillin	A (carboxylic acid)	2.9 (0.1)	15 (1)	+1	0.9969	0.90	318
	B (prim. amine)	6.9 (0.1)	-18 (2)	-1			
Labetalol	A (phenol)	7.26 (0.03)	15.3 (0.4)	+1	0.9999	0.14	9980
	B (second. amine)	9.35 (0.01)	-16.7 (0.2)	-1			
Procainamide	B (aniline)	2.70 (0.05)	38.2 (0.6)	+2	0.9987	0.38	2415
	B (tert. amine)	9.33 (0.02)	18.9[c]	+1			
Quinine	B (heterocycle)	4.31 (0.03)	38.0[c]	+2	0.9992	0.30	2634
	B (second. amine)	8.44 (0.03)	17.5 (0.3)	+1			
Cefalexin	A (carboxylic acid)	2.53 (0.06)	19.4 (0.9)	+1	0.9985	0.49	1305
	B (prim. amine)	7.01 (0.04)	-16.0[c]	-1			
Cefadroxil	A (carboxylic acid)	2.30 (0.08)	21 (2)	+1	0.9990	0.63	1236
	B (prim. amine)	7.07 (0.08)	-15 (1)	-1			
	A (phenol)	9.8 (0.2)	-29.1 (0.9)	-2			
Tetracycline	A (phenol)	3.19 (0.03)	17.7 (0.4)	+1	0.9996	0.25	4344
	A (phenol)	7.03 (0.05)	-9.8 (0.4)	-1			
	B (tert. amine)	9.5 (0.1)	-14.6[c]	-2			

[a] actual ionic mobility, in $m^2 \cdot V^{-1} \cdot s^{-1}$; [b] charge number of the protonated or dissociated forms of the drug; [c] directly measured

Electrophoretic data of the determinations is presented in Tables SI-1 to SI-7 of the supplementary information.

Two different pK_a values could be determined for all compounds, and three in case of cefadroxil and tetracycline. Statistical analysis shows that the fits of equation 6 to experimental data have coefficient of determination close to 1, and in general, low standard deviations associated with the pK_a values. However, this is usual in fits with a limited number of experimental points, especially if the actual ionic mobilities are also estimated. The highest standard deviation value corresponds to the third pK_a of cefadroxil and is caused by the small variation of mobility with pH when changing from the monocharged to the dicharged negative species of cefadroxil. In the Table 2 are also indicated the acid-base groups associated to the pK_a value. In the studied pH range procainamide and quinine are diprotic bases. Ampicillin, labetalol and cefalexin behave as zwitterionic compounds. Note that the only difference between cefalexin and cefadroxil is an extra phenolic group in cefadroxil (pK_{a3}). There is very good agreement between the pK_{a1} and pK_{a2} of both compounds, which correspond to identical acid-base moieties. Finally, tetracycline is a polyprotic compound with many acid-base groups. According to our data, it behaves as a triprotic compound in the pH range studied. A tentative assignation of the pK_a values according to ACD/Percepta software indicates that the first pK_a would belong to the phenolic group located in the amide ring, the second pK_a to the phenolic group located in the following aromatic ring, and the third pK_a, in the basic pH region, would belong to the tertiary amine.

As commented in the previous section, there is relatively few data related to pK_a values of drugs, especially polyprotic ones, determined at 37 ºC. From the seven drugs studied in this work we could only find the pK_a values of labetalol for comparison. They were determined potentiometrically by Avdeef et al.[32] in a 0.2M ionic strength media, and their values are 7.25 ± 0.01 and 9.00 ± 0.01. In order to compare our

results to the ones obtained in the literature, we have corrected the values from Avdeef et al. by the activity coefficients, obtaining pK_a values of 7.25 and 9.28, totally in accordance with the values obtained through the IS-CE method.

Conclusions

This work provides a set of internal standards for the high throughput determination of pK_a values of drugs at the biorelevant temperature of 37 ºC. The IS-CE method has several advantages over other methods of pK_a determination, but in this case it is of especial relevance that external measurement of the exact pH of the electrophoretic buffers and the pH electrode calibration at 37 ºC can be avoided. Accurate results can be obtained with only few electrophoretic runs. Comparison of the pK_a values at 25 ºC to the ones at 37 ºC for the set of internal standards indicates that in general pK_a decreases when temperature increase, although the magnitude of the change does not follow a general trend, but depends on the nature of the compound evaluated. The method has been applied successfully to the determination of pK_a values of polyprotic drugs, and very good accordance with available reference data has been observed.

Conflicts of interest

The authors declare no conflicts of interest.

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